

Delay in Feedback Repression by *Cryptochrome 1* Is Required for Circadian Clock Function

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SUMMARY

Direct evidence for the requirement of delay in feedback repression in the mammalian circadian clock has been elusive. *Cryptochrome 1* (*Cry1*), an essential clock component, displays evening-time expression and serves as a strong repressor at morning-time elements (E box/E' box). In this study, we reveal that a combination of day-time elements (D box) within the *Cry1*-proximal promoter and night-time elements (RREs) within its intronic enhancer gives rise to evening-time expression. A synthetic composite promoter produced evening-time expression, which was further recapitulated by a simple phase-vector model. Of note, coordination of day-time with night-time elements can modulate the extent of phase delay. A genetic complementation assay in *Cry1*^{-/-}:*Cry2*^{-/-} cells revealed that substantial delay of *Cry1* expression is required to restore circadian rhythmicity, and its prolonged delay slows circadian oscillation. Taken together, our data suggest that phase delay in *Cry1* transcription is required for mammalian clock function.

INTRODUCTION

Circadian clocks are thought to consist of autoregulatory loops with delayed transcriptional/translational feedback repression in which delayed expression of clock components is critical for maintaining circadian rhythmicity (Dunlap, 1999; Reppert and Weaver, 2002; Young and Kay, 2001). However, the underlying molecular mechanism giving rise to such delay remains

unknown, hindering formal validation of its biological relevance. In mammalian clocks, circadian transcriptional program is mediated through at least three clock-controlled DNA elements, morning-time (E box/E' box, or E/E' box: CACGT[G/T]) (Gekakis, 1998; Hogenesch et al., 1997; Ueda et al., 2005; Yoo et al., 2005), day-time (D box: TTA[T/C]GTAA) (Falvey et al., 1996; Ueda et al., 2005), and night-time elements (*Rev-Erb/ROR*-binding element, or RRE: [A/T]A[A/T]NT[A/G]GGTCA) (Harding and Lazar, 1993; Preitner et al., 2002; Ueda et al., 2002, 2005). The E/E' box-mediated transcriptional program has a critical role in the core autoregulatory loop of the mammalian circadian clock (Gekakis, 1998; Sato et al., 2006; Ueda et al., 2005). In this core loop, bHLH-PAS transcription activators such as BMAL1 and CLOCK form heterodimers that bind to E/E' box *cis*-elements in the promoter regions of their target genes, including the *Per* and *Cry* genes; CRYs, in turn, form repressor complexes that physically associate with the BMAL1/CLOCK complex to inhibit E/E' box-mediated transcription (Dunlap, 1999; Griffin et al., 1999; Kume et al., 1999; Reppert and Weaver, 2002; Young and Kay, 2001). Thus, the CRYs play an integral role in the circadian clock by "closing" the core negative feedback loop.

Although *Cry1*^{-/-} mice and their SCN slices display circadian rhythms at the organismal and tissue levels, respectively, dissociated *Cry1*^{-/-} SCN neurons and fibroblasts are largely arrhythmic. The issue of cell autonomy has been carefully examined in several recent studies (Brown et al., 2005; DeBruyne et al., 2007; Liu et al., 2007). By contrast to *Cry1*-deficient cells, dissociated *Cry2*^{-/-} SCN neurons and fibroblasts exhibit robust rhythmicity, implying that CRY2 cannot substitute for *Cry1* deficiency at the cellular level (Liu et al., 2007). Therefore, we focused on transcriptional regulation of *Cry1* gene.

CRY1 and its expression pattern play a pivotal role in the core autoregulatory loop. Either overexpression of CRY1 or interference of CRY1's repressor activity on E/E' box-mediated

transcription can abolish circadian transcriptional oscillations (Sato et al., 2006; Ueda et al., 2005). Remarkably, *Cry1* displays delayed gene expression relative to other genes with E/E' box elements (Ueda et al., 2002, 2005). Circadian expression of *Cry1* peaks at evening phases in the SCN (~CT12) (Ueda et al., 2002, 2005), which is much later than for typical morning-time E/E' box-regulated genes such as *Rev-Erb α* , and is intermediate between day-time D box- and night-time RRE-regulated genes such as *Per3* and *Bmal1*, respectively (Ueda et al., 2002, 2005). Dual roles of *Cry1* as a strong repressor for E/E' box activity and a time delay mediator fit well with the current model of the circadian clock, i.e., feedback repression with delay may depend on the unique mode of transcriptional regulation of *Cry1*.

Previous studies identified an E' box and an E box in *Cry1*'s regulatory region (Ueda et al., 2005; Fustin et al., 2009) and two RREs in its first intron (Ueda et al., 2005). In this study, we also identified additional D boxes in the promoter region and confirmed their functionality in conferring day-time expression (delayed phase relative to E box). We further discovered that a combination of the promoter containing E/E' boxes and D boxes with the first intron sequence of *Cry1* containing RREs generated delayed-phase expression of *Cry1*, in which the strength of night-time elements (RREs) can modulate the extent of phase delay. Of note, a simple phase vector model predicts that coordination between day-time and night-time elements can determine the extent of phase delay. Based on this model, we generated an array of *Cry1* constructs that display different phases, and these constructs were used in a genetic complementation assay to restore circadian oscillation in *Cry1*^{-/-}:*Cry2*^{-/-} cells. These experiments reveal that substantial delay of *Cry1* expression is required to restore single-cell level rhythmicity and that prolonged delay of *Cry1* expression can slow circadian oscillation. These results suggest that phase delay in transcriptional feedback repression is required for mammalian clock function.

RESULTS

Cry1 Promoter Confers Phase and Amplitude Intermediate between Those Conferred by E/E' Box and D Box Circadian Elements

To examine *Cry1* promoter activity, we generated a reporter construct, P(*Cry1*)-*Luc*, in which a 1.5 kbp DNA fragment containing the *Cry1* promoter was fused to the *Luciferase* (*Luc*) gene. *Cry1* promoter-driven bioluminescence reached its peak at circadian time (CT) 9.60 \pm 0.11 (n = 3, mean \pm standard deviation), which was rather close to that of a D box-P(SV40)-*Luc* reporter harboring three tandem repeats of D boxes fused to an SV40 promoter (CT10.42 \pm 0.16), and delayed > 6 hr relative to an E' box-P(SV40)-*Luc* reporter harboring three tandem repeats of E' boxes fused to an SV40 promoter (CT3.53 \pm 0.04) (Figure 1A and Figure S1A and Table S1 available online). On the other hand, the *Cry1* promoter produced a higher-amplitude rhythm than that of the D box-P(SV40)-*Luc* reporter (Figure 1B). The amplitude of E' box-driven bioluminescence rhythms (Figure 1B, E' box-P(SV40)-*Luc*) was even higher than those driven by the *Cry1* promoter. These data place the *Cry1* promoter intermediate between D box and E' box in both phase and amplitude

of driven rhythms and suggest that the *Cry1* promoter might contain both D box and E' box elements.

D Box in *Cry1* Promoter Confers Phase Delay and Day-Time Expression

We investigated the genomic sequences of the *Cry1* promoter and found five highly conserved regions, of which two sequences (5'-TTCAGAAA-3' and 5'-AAACGTGA-3') most closely resemble a D box according to position weight matrix analysis. Interestingly, these sequences overlap with the conserved E' box sequences in the promoter region (Figure 1C). We designated this region of the *Cry1* promoter as a *Cry1*proD element and constructed a *Cry1*proD-P(SV40)-*Luc* reporter by fusing three tandem repeats of this element to an SV40 promoter. NIH 3T3 cells transiently transfected with this construct showed circadian oscillation of bioluminescence with a peak at day-time (CT12.37 \pm 0.05, n = 3; Figure 1D, Figure S1B, and Table S1) and a relative amplitude between those of E' box and D box constructs (Figure 1E). Because the E' box and two putative D boxes in *Cry1*proD element overlap, it is not practical to isolate each CCE for analysis individually. Instead, we tested whether clock factors involved in E' box- or D box-mediated transcription could activate or repress the *Cry1*proD element. Cotransfection of E' box activators BMAL1/CLOCK strongly induced not only E' box, but also *Cry1*proD activity in NIH 3T3 cells (Figure 1F). Cotransfection of a D box repressor E4BP4 inhibited BMAL1/CLOCK induction of *Cry1*proD activity in a dose-dependent manner (Figure 1F). Interestingly, the E' box repressor CRY1 also inhibited this induction (Figure S1C), and the D box activators DBP, HLF, and TEF (Mitsui et al., 2001) also induced *Cry1*proD activity (Figure S1D). These results suggest that *Cry1*proD is regulated by classical transcriptional regulators of both D box and E/E' box activities, consistent with the observation that bioluminescence rhythms driven by the *Cry1* promoter display properties intermediate between those driven by D box and E' box constructs.

To confirm that *Cry1* expression is delayed relative to E/E' box activity, we measured temporal mRNA profiles of endogenous *Per2*, *Bmal1*, and *Cry1* in NIH 3T3 cells expressing a P(*Cry1*)-*Luc* or a P(*Per2*)-*Luc* reporter (Figure 1G, left). As shown, the phase of *Luc* mRNA driven by the *Per2* promoter was almost the same as that of endogenous *Per2* mRNA (Figure 1G, right). Furthermore, the phase of *Luc* mRNA driven by the *Cry1* promoter was delayed relative to those of endogenous *Per2* mRNA or *Luc* mRNA driven by the *Per2* promoter, with a phase difference of ~1–2 hr. These results further support the notion that functional D boxes in the *Cry1* promoter contribute to phase delay of *Cry1* expression.

Cry1 Intron Acts as an Enhancer to Confer Phase Delay

In addition to the phase delay caused by D boxes within the *Cry1* promoter, we also observed further phase delay of the endogenous *Cry1* mRNA by at least 2 hr relative to *Luc* mRNA driven by the *Cry1* promoter (Figure 1G). The endogenous *Cry1* mRNA displayed ~3–4 hr phase delay relative to endogenous *Per2* mRNA and ~7–8 hr advance relative to *Bmal1* mRNA. This observation is consistent with previous reports (Baggs et al., 2009; Etchegaray et al., 2003; Liu et al., 2008; Preitner

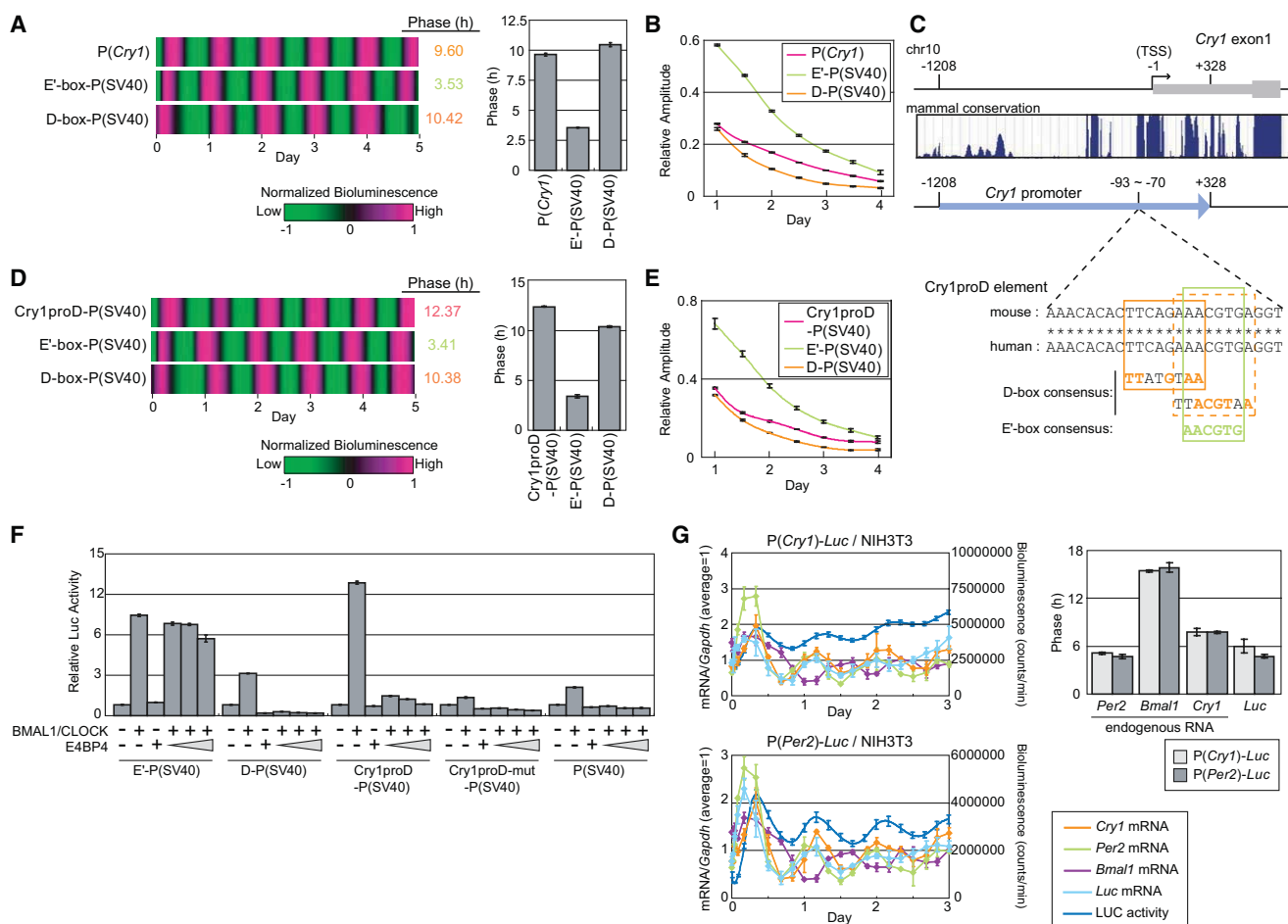


Figure 1. The *Cry1* Promoter Contains Both E/E' Box and D Boxes

(A) The phases of circadian transcriptional activities induced by P(*Cry1*), E' box-P(SV40), and D box-P(SV40) promoters. Each promoter was fused with a *Luciferase* reporter gene (*Luc*) and transiently transfected into NIH 3T3 cells. Time series of bioluminescence expression were recorded in real time using a photomultiplier tube (PMT). Heatmaps represent average promoter activities from three independent samples. Raw data were detrended for baseline and amplitude and then scaled into a range of -1 to 1 (left). Phases were estimated from the time series data by fitting a cosine wave (right).

(B) The relative amplitudes of circadian transcriptional activities induced by P(*Cry1*), E' box-P(SV40), and D box-P(SV40) promoters.

(C) E' box and its overlapping D boxes in *Cry1* promoter. Genomic positions relative to the transcription start site (TSS, designated also as "-1") of *Cry1* gene are indicated along with evolutionary conservation scores among mammalian species. Colored letters indicate nucleotides matching the consensus sequence of D box and E' box.

(D) The phases of circadian transcriptional activities induced by Cry1proD-P(SV40), E' box-P(SV40), and D box-P(SV40) promoters. The experiment was performed as in (A).

(E) The relative amplitudes of circadian transcriptional activities induced by Cry1proD-P(SV40), E' box-P(SV40), and D box-P(SV40) promoters.

(F) The Cry1proD element can be activated by BMAL1/CLOCK and repressed by E4BP4. Each of the indicated promoters was fused to a *Luciferase* reporter gene and transiently transfected into NIH 3T3 cells. Relative *Luciferase* activity for each promoter was scaled so that the activity without transcriptional regulation is normalized to 1.

(A-F) Data are representative of two independent experiments. Error bars represent SD determined from three measurements for each sample (n = 3).

(G) mRNA expression patterns of endogenous *Cry1*, *Per2*, and *Bmal1* and exogenous *Luciferase* (*Luc*). NIH 3T3 cells were transfected with P(*Cry1*)-*Luc* or P(*Per2*)-*Luc* reporter. Relative mRNA levels of each gene were measured. In parallel, transcriptional activities of P(*Cry1*) and P(*Per2*) promoters were monitored by bioluminescence recording. Phases of measured rhythm are indicated on the right. (Right) Error bars represent SEM (n = 3). (Left) Error bars represent SD (n = 3). See also Figure S1 and Table S1.

et al., 2002; Sato et al., 2006; Ueda et al., 2005), which have speculated that a phase delay would be generated by two functional RREs present in the intron regions of *Cry1* (Ueda et al., 2005). To provide experimental evidence for the mechanism of further phase delay of *Cry1* expression, we focused on one of the highly conserved regions of the *Cry1* gene—the first intron,

which contains two RREs, designated here as R1 and R2 (Figure 2A). These RREs are highly conserved and aligned in a head-to-head arrangement, perfectly matched to the consensus RRE sequence ([A/T]A[A/T]NT[A/G]GGTCA). We cloned a 1.03 kbp fragment containing the conserved intronic RREs and inserted it into the P(*Cry1*)-*Luc* reporter plasmid

to generate a P(*Cry1*)-*Cry1* intron 1.03k-*Luc* reporter. Cells expressing this reporter displayed a bioluminescence peak at $CT14.62 \pm 0.20$, whereas absence of the 1.03 kbp intron sequence resulted in a peak at $CT10.51 \pm 0.30$, a difference of ~ 4 hr (Figure 2B, Figure S2A, and Table S1). We next focused on a highly conserved region of 336 bp within the 1.03 kbp intron sequence for further analysis. Whereas cells expressing a P(*Cry1*)-*Cry1* intron $\Delta 336$ -*Luc* reporter exhibited a peak at $CT10.37 \pm 0.24$, those expressing P(*Cry1*)-*Cry1* intron 336-*Luc* peaked at $CT14.32 \pm 0.23$ (Figure 2B, Figure S2A, and Table S1), a 4 hr phase delay. Thus, *Cry1* first intron sequences containing RREs likely underlie the delayed phase of *Cry1* expression. In addition, their effects appear to be independent of locations (Figure 2C, Figure S2B, and Table S1), suggesting that this sequence functions as a transcriptional enhancer.

Next, we analyzed the regulatory regions for the presence of their corresponding transcription factors in vivo using chromatin immunoprecipitation (ChIP) assays with time series samples from mouse liver. Chromatin from wild-type, *Dbp*^{-/-} (Lopez-Molina et al., 1997), or *Rev-Erb* α ^{-/-} (Preitner et al., 2002) mice was immunoprecipitated by anti-BMAL1, anti-DBP, or anti-REV-ERB α antibodies (Figure 2D). The levels of BMAL1 and DBP binding to *Cry1*proD displayed circadian oscillation in wild-type, *Dbp*^{-/-}, or *Rev-Erb* α ^{-/-} mice, whereas DBP binding in *Dbp*^{-/-} mice was significantly reduced ($p < 0.01$ by two-way ANOVA) with residual signals potentially deriving from TEF and/or HLF binding to the same element. On the other hand, no significant reduction was observed for the binding of DBP to this region in *Rev-Erb* α ^{-/-} mice. The level of REV-ERB α binding to the *Cry1* first intron region also displayed circadian oscillation in wild-type and *Dbp*^{-/-} mice, whereas it was significantly reduced in *Rev-Erb* α ^{-/-} mice ($p < 0.01$ by two-way ANOVA). The levels of BMAL1 binding to the *Dbp* promoter region displayed circadian oscillation, whereas there was only background binding of DBP and REV-ERB α to this region. This result confirmed that BMAL1 and DBP bind to the *Cry1* promoter region, and REV-ERB α binds to the *Cry1* first intron region. The peak binding time of each transfactor is consistent with previous reports of its in vivo binding or its nuclear accumulation (Lopez-Molina et al., 1997; Mitsui et al., 2001; Preitner et al., 2002; Ripperger and Schibler, 2006).

In addition to the biochemical interaction between the *Cry1* promoter and D box *trans*-regulators described above, we also examined the role of the D box using genetic approaches; we measured mRNA expression patterns from time course liver samples of triple-knockout mice of PAR bZip genes (*Tef*, *Hlf*, and *Dbp*) (Gachon et al., 2004). Although these mice displayed normal circadian behavior (possibly due to compensation rendered by posttranslational mechanisms intracellularly and/or intercellular coupling of clock cells in vivo) (Gachon et al., 2004; Lee et al., 2001; Liu et al., 2007), we found that *Cry1*'s circadian expression level was different from wild-type and its peak of expression delayed (Figure 2E and Figure S2C), whereas those of other measured clock genes (*Bmal1*, *RevErb* α , and *Per1*) were not. Importantly, the observed peak delay was reproducible and significant in three independent experiments ($p < 0.01$ by two-way ANOVA). These results further confirm that PAR bZip genes are important for the proper phase of expression of *Cry1*.

Strength of Intronic RREs Correlates with Phase Delay

To determine whether the strength of RREs in 336 bp of *Cry1* first intron sequence correlates with the phase delay, we generated an array of intron sequences harboring mutant RREs, including deletion, mutation, and inversion of the two RREs (Figure 3A). We inserted these mutant intron sequences into P(SV40)-*Luc* vector to generate an array of P(SV40)-*Cry1* intron 336-*Luc* reporter constructs. As one measurement for the strength of intronic RREs, we first examined transcriptional activation of these constructs by ROR α , an activator of RRE, in a reporter assay. We found that induced *Luciferase* activities varied significantly among constructs, ranging from strong induction by wild-type RRE to almost no induction by double-mutant or deleted RREs (R1 and R2) (Figure 3B). These results indicate that the RREs within the intron sequence are functionally responsive to ROR α and that intronic RREs of various strengths can be obtained from different RRE mutations.

As an independent measurement for the strength of intronic RREs, we next examined the amplitude of circadian oscillations expressed by these constructs in reporter rhythm assays (Figure S3A and Table S1). Rhythm amplitude was low when an intron sequence of low ROR α responsiveness was used to drive reporter expression and high when an intron sequence of high ROR α responsiveness was used (Figures 3B and 3C). Overall, there was a significant positive correlation between the two measurements for the strength of intronic RREs: ROR α responsiveness and rhythm amplitude among the intronic RRE mutants ($r^2 = 0.95$, $p < 0.01$; Figure 3D).

These mutant intron sequences allowed us to analyze quantitatively the role of intronic RREs in the phase delay mechanism. Specifically, we examined how intronic RRE mutation affects phase delay using a reporter rhythm assay (Figure 3E, Figure S3B, and Table S1). We found that the observed phase delay significantly correlated with the first measurement for the strength of the intronic RRE mutants, i.e., ROR α responsiveness ($r^2 = 0.82$, $p < 0.01$; Figure 3F, top). Similarly, phase delay also correlated well with the second measurement for the strength of the intronic RRE mutants, i.e., the rhythm amplitude ($r^2 = 0.90$, $p < 0.01$; Figure 3F, bottom). Taken together, these data suggest that the strength of RREs correlates with the phase delay, further corroborating our finding that the RREs in the *Cry1* intron act as an enhancer to further delay the phase conferred by *Cry1* promoter.

Combination of Day- and Night-Time Elements Produces Evening Phase Control

Given that the delayed expression of *Cry1* is a combined effect of its promoter and intron, we sought to understand whether this combinatorial effect is a general design principle in the circadian transcriptional network or a mechanism unique to the transcriptional regulation of *Cry1*. We first asked whether the phase of endogenous *Cry1* expression could be synthesized using an artificial promoter in clock cells. We constructed three sets of reporters, with each harboring one of the three CCEs (i.e., E/E' box, D box, and RRE) in the presence or absence of the RRE-containing intron sequence from the *Cry1* gene (Figures 4A and 4B). Real-time bioluminescence recording of transfected NIH 3T3 cells showed that the RRE-containing *Cry1* intron sequence, as expected, did not dramatically alter the phase of

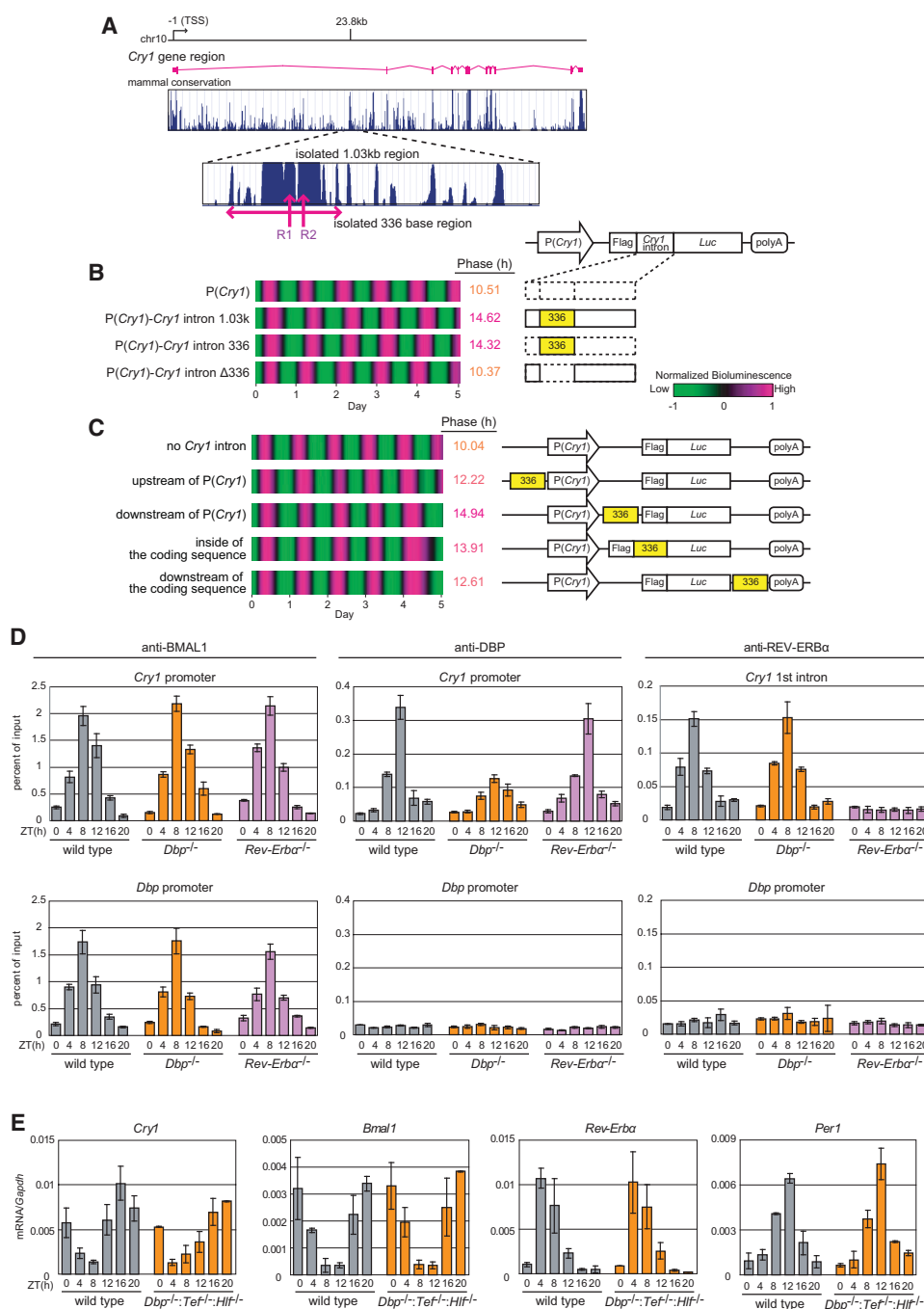


Figure 2. Cry1 Intron Acts as an Enhancer to Confer Phase Delay

(A) The first intron of *Cry1* contains RRE sequences. The marked 1.03 kbp and 336 bp of *Cry1* first intron sequence, which are highly conserved in mammals, were cloned and examined in this study. Two RREs are indicated as R1 and R2, respectively.

(B) *Cry1*'s first intron confers phase delay. The *Cry1* promoter was combined with the *Cry1* 1.03 kbp intron, 336 bp intron, or *Cry1* intron Δ336 deletion mutant to generate composite promoters. The experiment was performed as in Figure 1A.

(C) *Cry1*'s first intron sequence confers phase delay independently of its location. The 336 bp of *Cry1* intron sequence was inserted upstream or downstream of the *Cry1* promoter and inside or downstream of the coding sequence. Data are representative of two independent experiments (B and C).

(D) Binding of BMAL1 (an E/E' box regulator) and DBP (a D box regulator) to the *Cry1* promoter region and REV-ERBα (a RRE regulator) to the *Cry1* first intron region in vivo. Chromatin from wild-type (gray), *Dbp*^{-/-} (orange), or *Rev-Erba*^{-/-} (purple) mice was prepared at 4 hr intervals from mice held in a 12 hr light/12 hr dark cycle (LD 12:12). The binding of each regulator to its regulatory region was analyzed by ChIP with the indicated antibodies. Note that DBP binding in *Dbp*^{-/-} mice was significantly reduced ($p < 0.01$ by two-way ANOVA), with residual signals potentially deriving from TEF and/or HLF binding. Specific TaqMan probes

RRE-mediated reporter expression, albeit with an increase in amplitude (Figure S4). The intron sequence sometimes caused double peaks for the E' box-driven rhythms (Figure 4A and Figure S4). When these rhythms were fitted to a circadian cosine curve, we observed a reduction of the relative amplitude and a slight but reproducible phase advance (Figure 4A, Figure S4, and Table S1). Importantly, the combination of D box in the promoter and RRE-containing *Cry1* intron sequence conferred a substantial phase delay of > 5 hr ($CT14.48 \pm 0.21$) when compared to the D box alone ($CT9.31 \pm 0.16$) (Figure 4, Figure S4, and Table S1). It is important to note that our result indicates that E/E' boxes are dispensable for the generation of delayed-phase expression of *Cry1*. This is because the synthetic composite "D box + RRE" promoter (i.e., a combination of a synthetic D box-driven promoter and RRE-containing *Cry1* intron sequence) lacks functional E boxes, unlike the *Cry1* promoter. Thus, the D box and the RRE can combine to generate a distinct intermediate phase.

We were able to recapitulate these experimental measurements in a simple model using "phase vectors." A phase vector represents phase and amplitude of the oscillation as direction and length of the vector in polar coordinates. In this way, the combination of two oscillations can be represented by the vector sum of two corresponding phase vectors (Extended Experimental Procedures). We plotted measured oscillations (CCE without intron sequence and intron sequence without CCE) and obtained the summed phase vector of the CCE-intron sequence combinations (Figure 4B, left three circles). Interestingly, the summed phase vectors corresponded well with the measured oscillations (Figure 4B, rightmost). These results support the notion that combining two CCEs that otherwise function independently can be a general mechanism for generation of new phases and, more specifically, the combined phase may be predicted, to a first-order approximation, by a vector sum.

Delayed Expression of *Cry1* Restores Circadian Rhythmicity in *Cry1*^{-/-}:*Cry2*^{-/-} Cells

To address the functional importance of the RRE-mediated phase delay, we employed cell-based genetic complementation, testing for phenotypic rescue in arrhythmic *Cry1*^{-/-}:*Cry2*^{-/-} cells. We hypothesized that, if phase delay is an important property of *Cry1*, its delayed expression, peaking at evening-time, should restore circadian oscillations in these cells. To test this hypothesis, we established mouse embryonic fibroblasts from *Cry1*^{-/-}:*Cry2*^{-/-} double-knockout mice (van der Horst et al., 1999). Similar to negative control (Figure 5A, without *Cry1*), *Cry1* expression driven only by the *Cry1* promoter, P(*Cry1*), did not rescue circadian oscillations in these cells (Figure 5A, P(*Cry1*)). However, when *Cry1* expression was regulated by

P(*Cry1*)-*Cry1* intron 336, which contains the *Cry1* promoter and the RRE-containing 336 bp of *Cry1* intron sequence, its exogenous expression restored circadian rhythmicity in these cells, with a period length of 26.73 ± 0.19 hr (Figure 5A, *Cry1* intron 336). The observed rescue capability was independent of the CRY1 protein level, vector type, or method of DNA delivery (Figures S5A and S5B). Taken together, these results demonstrate that delay of *Cry1* expression, conferred by the *Cry1* intron, is required for rescue of circadian rhythmicity.

To further assess the contribution of delayed *Cry1* expression to the rescued circadian oscillation, we tested the rescue capability of the intronic RREs mutants that possess different RRE strengths, as described above (Figure 3 and Figure 5A, nine panels on the right, and Table S2). The ability of the intronic RRE mutants to rescue rhythmicity, represented as amplitude of circadian oscillations, significantly correlated with the strength of intronic RREs, as measured by bioluminescence levels derived from P(SV40)-*Cry1* intron 336-*Luc* ($r^2 = 0.87$, $p < 0.01$; Figure 5B, left). Similarly, the rescue capability also correlated with another measurement of strength of intronic RREs, i.e., ROR α responsiveness of the intronic RRE mutants ($r^2 = 0.97$, $p < 0.01$; Figure 5B right). More directly, the rescue capability correlated with the phase delay conferred by the intronic RRE mutants that was measured in bioluminescence rhythms of P(*Cry1*)-*Cry1* intron 336-*Luc* ($r^2 = 0.71$, $p < 0.01$; Figure 5C). It should be noted that the rescue capability in these experiments does not correlate with either amplitude or basal bioluminescence levels of P(*Cry1*)-*Cry1* intron 336-*Luc* (Figure S5C), suggesting that the rescue capability is most likely attributable to the delayed phase of *Cry1* expression conferred by RREs. To directly confirm this, we demonstrated that the pure RREs, when combined with *Cry1* promoter, rescued circadian rhythmicity in *Cry1*^{-/-}:*Cry2*^{-/-} cells, whereas *Cry1* promoter alone could not reliably rescue rhythms (Figure S5D and Figure 5).

The *Cry1*-rescued *Cry1*^{-/-}:*Cry2*^{-/-} cells (a *Cry2* knockout, in essence) showed a rather long period length of ~ 27 hr (Figure 5A), which is consistent with previous reports showing that *Cry2*^{-/-} single-knockout cells display long periods compared to wild-type cells (~ 24 – 25 hr) (Liu et al., 2007). We confirmed that genetic complementation of *Cry1* in *Cry1*^{-/-}:*Cry2*^{-/-} cells recapitulates the circadian phenotype in *Cry2* single-knockout cells, thus phenotypically validating the *Cry1* rescue assay (Figure S5E and Table S3).

Cry1 Phase Delay Modulates Circadian Period Length

The genetic complementation assay expressing *Cry1* of various phases revealed that delay of *Cry1* expression is required to restore circadian rhythmicity, consistent with the proposed design principle for circadian clocks, i.e., transcriptional/translational

were used to detect the *Cry1* promoter region, the *Cry1* first intron region, or the *Dbp* promoter region (control region). ZT, Zeitgeber time. Mean and SD represent three ChIP experiments.

(E) Circadian expression profiles of *Cry1*, *Bmal1*, *Rev-Erb α* , and *Per1* in vivo. RNA from liver of wild-type (gray) or *Dbp*^{-/-}:*Tef*^{-/-}:*Hlf*^{-/-} (orange) mice was prepared at 4 hr intervals from mice held in LD 12:12 cycle. Relative mRNA levels of each gene were measured. Note that *Cry1* expression in *Dbp*^{-/-}:*Tef*^{-/-}:*Hlf*^{-/-} mice was different from wild-type and its peak delayed. All RNA samples were normalized to *Gapdh* mRNA accumulation. Mean and SEM from two pools of three mice each per time point. The representative data from three independent experiments was shown. The observed peak delay was reproducible and significant in three independent experiments ($p < 0.01$ by two-way ANOVA).

See also Figure S2 and Table S1.

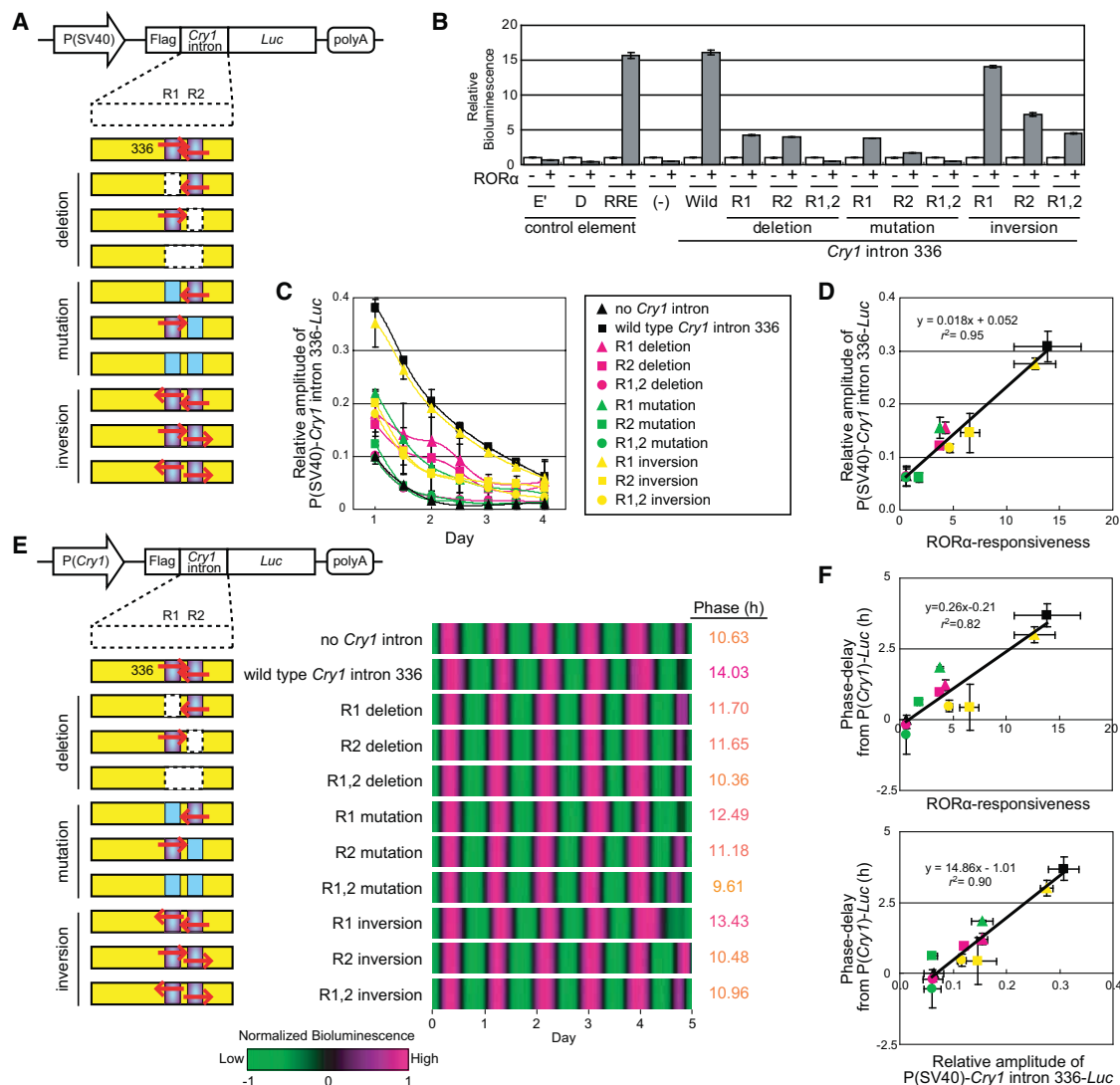


Figure 3. The Strength of Intronic RREs Correlates with Phase Delay

(A) Mutant reporter constructs derived from P(SV40)-*Cry1* intron 336-*Luc*. P(SV40)-*Cry1* intron 336-*Luc* contains the 336 bp of *Cry1* intron sequence (wild-type); the RREs or R1/R2 within the *Cry1* intron sequence were altered to generate three sets of *Cry1* intron sequence mutants: deletions, mutations, and inversions. Red arrow indicates the direction of R1 and R2. Purple, light blue, and white rectangles represent wild-type, mutated, and deleted RREs, respectively.

(B) RORα responsiveness of mutated intron sequences. Each reporter construct in (A) was transiently transfected into NIH 3T3 cells in the absence (-) or presence (+) of RORα. Luciferase activities were scaled so that basal activity without RORα was 1.

(C) Relative amplitudes of circadian transcriptional activities induced by constructs presented in (A).

(D) Correlation between two measurements for the strength of intronic RREs, the RORα responsiveness (B), and the relative rhythm amplitude (C) of mutated intron sequences.

(E) Mutant reporter constructs derived from P(Cry1)-*Cry1* intron 336-*Luc* and their phases. The SV40 promoter in P(SV40)-*Cry1* intron 336-*Luc* was replaced with *Cry1* promoter P(Cry1) to generate P(Cry1)-*Cry1* intron 336-*Luc*. The RRE mutations in P(Cry1)-*Cry1* intron 336-*Luc* are the same as in P(SV40)-*Cry1* intron 336-*Luc* constructs in (A).

(F) Phase delay correlates with two measurements for the strength of intronic RREs, the RORα responsiveness and the relative rhythm amplitude. The RORα responsiveness presented in (B) (top) and relative rhythm amplitudes presented in (C) (bottom) of mutated intron sequences are plotted against phase delay of P(Cry1)-*Cry1* intron 336-*Luc* activity relative to P(Cry1)-*Luc* activity presented in (E).

Data are representative of two independent experiments (B, C, and E). Error bars represent SD (n = 3) (B and C). Mean and SD (error bar) of two independent experiments are shown (each experiment contains three samples; n = 3 unless otherwise indicated in Table S1) (D and F). See also Figure S3 and Table S1.

feedback repression with delay. This design principle further predicts that *Cry1* expression with a more prolonged delay can slow circadian oscillations. To test this prediction, we first attempt-

ted to generate constructs expressing *Cry1* with prolonged delays. According to the phase-vector model described above (Figure 4B), we should be able to generate evening-to-night

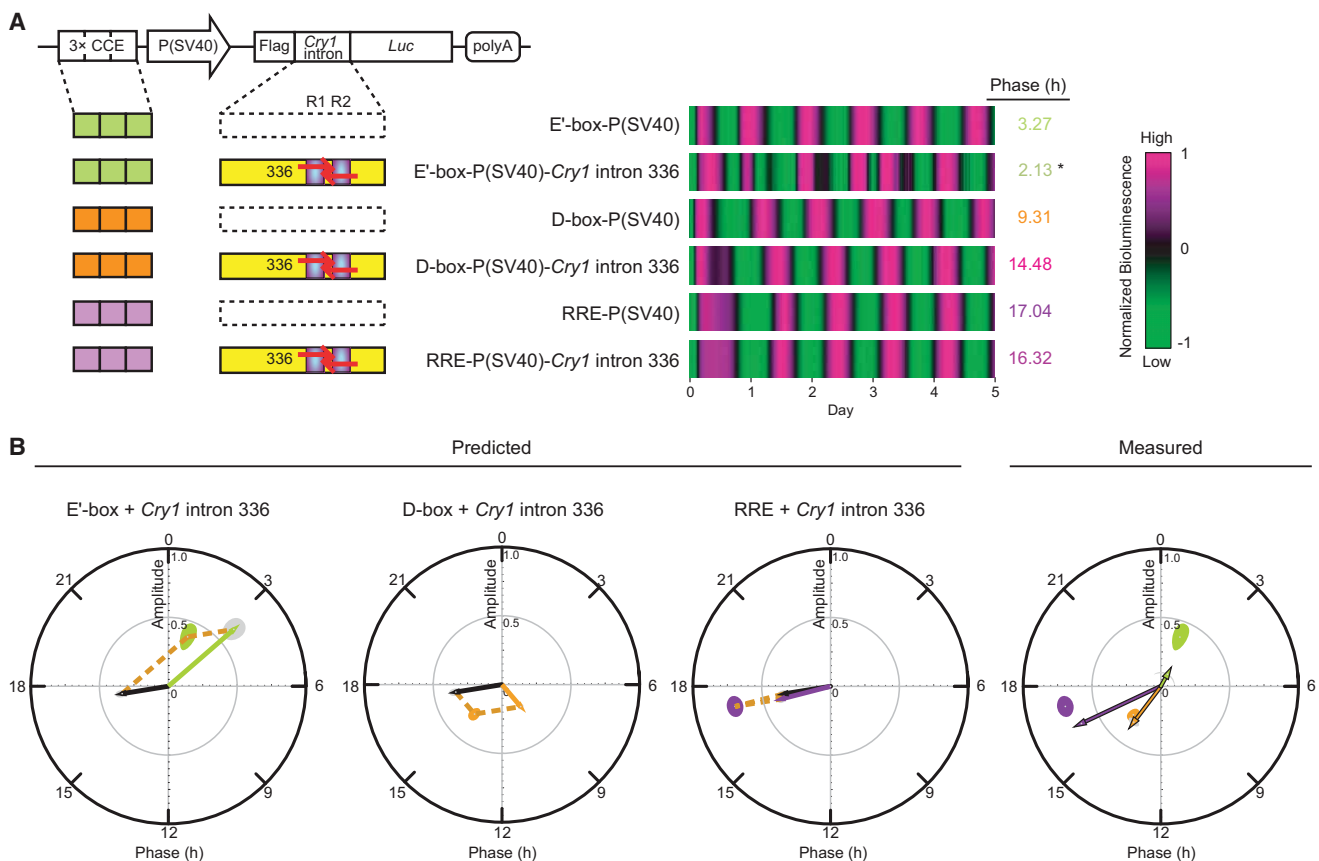


Figure 4. Combination of Intronic RREs with Known Circadian *cis*-Elements Gives Rise to Emergent Phases that Can Be Predicted by Phase Vectors

(A) Combination of the *Cry1* intron sequence with known CCEs gives rise to emergent phases. A promoter was constructed by inserting 3 × E' box, 3 × D box, or 3 × RRE sequences in the upstream of P(SV40). In the reporter construct, *Luciferase* expression was under the control of the 3 × CCE-P(SV40) promoter in the absence or presence of the 336 bp of *Cry1* intron sequence. The experiment was performed as in Figure 1A. Phases were estimated by fitting a cosine wave with circadian period corresponding to maximum autocorrelation of the time series using detrended bioluminescence data. This method allowed phase estimation of even distorted wave form expressed by E' box + *Cry1* intron sequence (marked by asterisk).

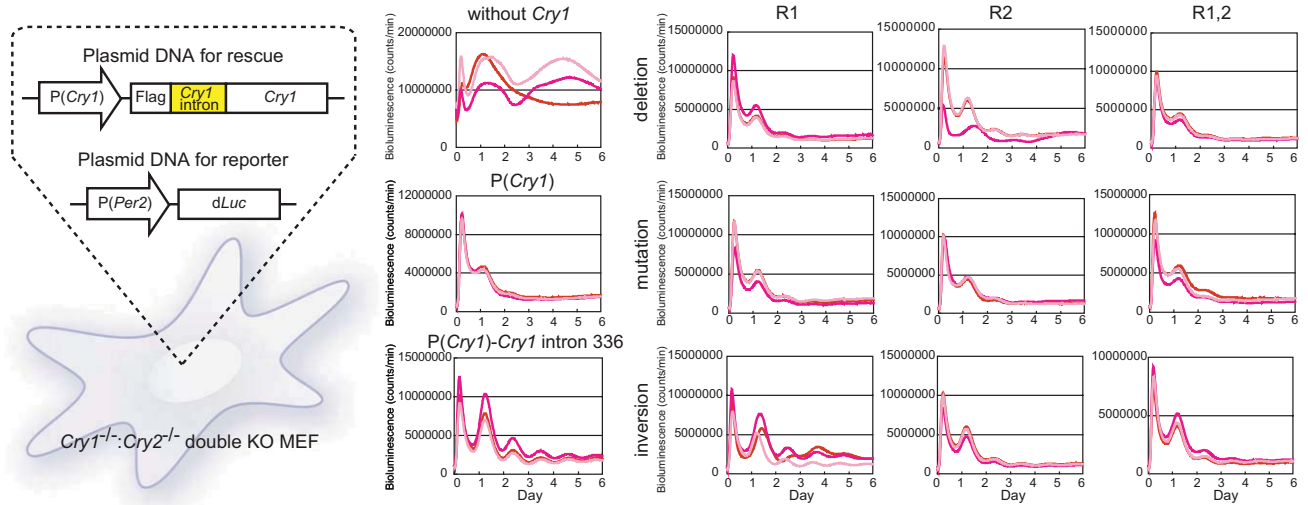
(B) A phase vector model recapitulates the emergent phases. The phase vector of each CCE (E' box, green arrow; D box, orange arrow; RRE, purple arrow) and *Cry1* intron sequence (black arrow) and the vector sum of the two phase vectors (center of colored ellipsoid) are plotted in the polar coordinate (left three circles). The ellipsoidal disk represents 95% confidence region. The phase vectors (colored arrows of black border) represent measured circadian transcriptional activities induced by the combined regulation of *Cry1* intron sequence and each CCE (rightmost circle).

Data are representative of two independent experiments. See also Figure S4 and Table S1.

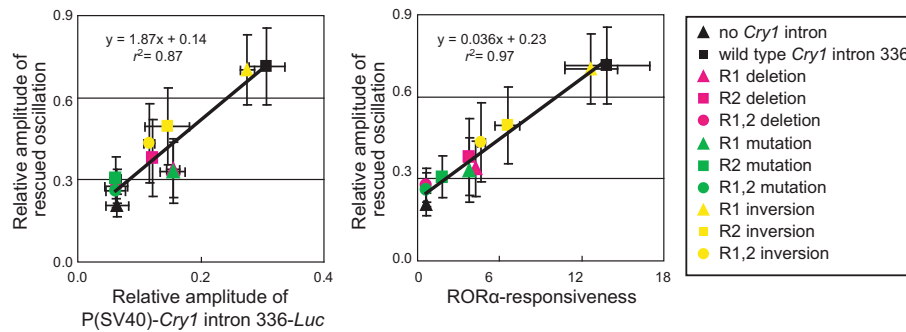
expression with a more prolonged delay by weakening the day-time promoter but keeping a constant strength of the night-time enhancer of *Cry1* intron sequence. Therefore, we generated an array of day-time promoters with various strengths of D boxes, containing 1, 2, or 3 tandem repeats of D boxes or *Cry1*proD elements; we confirmed that these day-time promoters displayed day-time phased bioluminescence rhythms of various relative amplitudes, as expected (Figure 6A, Figure S6A, and Table S1). We then generated another set of constructs by combining these day-time promoters with the *Cry1* intron sequence (Figure 6B, Figure S6B, and Table S1). These constructs displayed evening-to-night phases of bioluminescence rhythms (Figure 6B, Figure S6B, and Table S1). Importantly, there was a significant correlation between the observed phases and the predicted phases from the simple phase-vector model ($r^2 = 0.77$, $p < 0.01$; Figure 6C, rightmost panel).

Next, we asked whether evening-to-night *Cry1* expression with prolonged delay could slow circadian oscillations (Figure 6D and Table S3). Interestingly, the periods of rescued circadian oscillations ranged from 27 to 31 hr. In particular, period length correlated with the delay prolonged by weakening the day-time promoter: the more the *Cry1* phase was delayed, the longer the rescued period ($r^2 = 0.81$, $p < 0.01$; Figure 6E and see also Figure S6C). We also confirmed that the period length did not significantly correlate with either amplitude or basal activity of *Cry1* expression by using a different constitutive promoter (Figure S6D and Table S3). In addition, CRY1 protein level was not responsible for the changes in rescued period (Figure S6E). These results showed that *Cry1* expression with a prolonged delay slows circadian oscillations, further supporting the proposed design principle of circadian clocks—transcriptional/translational feedback repression with delay.

A



B



C

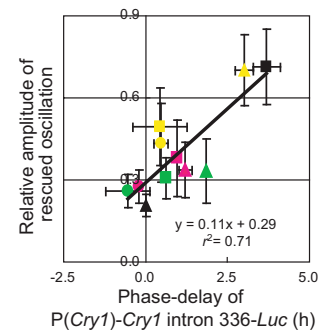


Figure 5. Delayed Expression of *Cry1* Restores Circadian Rhythmicity in *Cry1*^{-/-}:*Cry2*^{-/-} Cells

(A) Genetic complementation of *Cry1* rescues circadian oscillation in *Cry1*^{-/-}:*Cry2*^{-/-} cells. A schematic diagram of *Cry1* rescue constructs is shown on the left. The composite promoter contains P(*Cry1*) and the 336 bp *Cry1* intron sequence of wild-type or a mutant (deletion, mutation, or inversion of the R1 and R2 sequences as in Figure 3), which controls *Cry1* expression. *Cry1* rescue constructs were each cotransfected with a destabilized *Luciferase* reporter construct, P(*Per2*)-dLuc, into *Cry1*^{-/-}:*Cry2*^{-/-} mouse embryonic fibroblast cells (left), followed by bioluminescence recording. Whereas mock-transfected *Cry1*^{-/-}:*Cry2*^{-/-} cells were completely arrhythmic and those expressing P(*Cry1*)-*Cry1* were only transiently rhythmic during the first 2 days of recording, P(*Cry1*)-*Cry1* intron 336-*Cry1* expression restored circadian oscillation with a period length of 26.73 ± 0.19 hr (bottom in the center column). Rescue effects varied among the intronic RRE mutants (right nine panels). Data are representative of two independent experiments.

(B) Relative amplitude of rescued circadian oscillation correlates with the strength of intronic RREs. The relative amplitudes of rescued oscillation are plotted against two measurements for the strength of intronic RREs, the relative amplitudes of P(SV40)-*Cry1* intron 336-*Luc* oscillation presented in Figure 3C, and the ROR α responsiveness presented in Figure 3B.

(C) Relative amplitude of rescued circadian oscillation correlates with phase delay. The relative amplitudes of rescued oscillations are plotted against the phase delay of various P(*Cry1*)-*Cry1* intron 336-*Luc* activities relative to P(*Cry1*)-*Luc* activity presented in Figure 3E.

Mean and SD (error bar) of two independent experiments are shown (each experiment contains three samples; $n = 3$ unless otherwise indicated in Table S1). See also Figure S5 and Table S2.

Single-Cell Analysis Confirms the Importance of *Cry1* Phase Delay in Feedback Repression

Arrhythmic phenotypes observed in population of cells might be due to rapid damping of individual cells or lack of synchronization among individual cells. To discriminate between these possibilities, we monitored bioluminescence levels in real time at the level of single-cell resolution (Sato et al., 2006; Ukai et al., 2007). As with whole-well assays, single-cell analysis showed that most individual cells expressing *Cry1* with a normal delay, driven by the intron sequence containing wild-type RREs, were robustly rhythmic, with a circadian period of 26.77 ± 0.12 hr

(Figures 7A and 7B and Table S3), whereas most cells expressing *Cry1* without delay, driven by an intron sequence harboring mutated RREs, were arrhythmic (Figure 7B and Movie S1). Moreover, individual cells expressing *Cry1* with a prolonged delay driven by the *Cry1* intron sequence alone (i.e., in the absence of *Cry1* promoter) displayed long circadian periods of up to 32.00 ± 0.58 hr (Figures 7A and 7B and Table S3). The circadian oscillations in Figure 7A with delayed *Cry1* expression were statistically significant ($p < 0.01$ by autocorrelation) and reproducible in different series of experiments. Thus, single-cell analysis confirmed the circadian phenotypes observed in

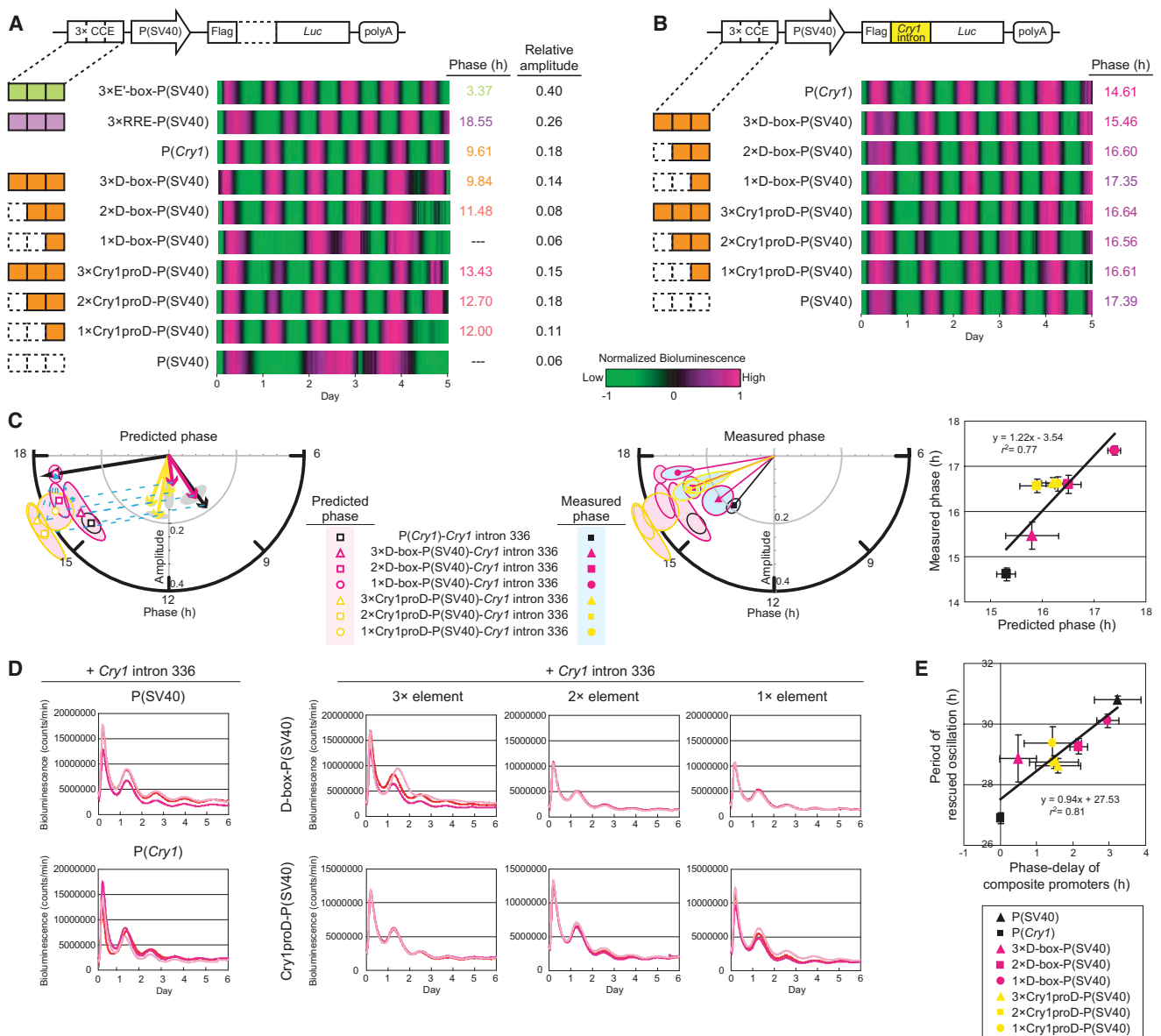


Figure 6. Prolonged Delay of *Cry1* Expression Slows Circadian Oscillations in *Cry1*^{-/-}:*Cry2*^{-/-} Cells

(A) Promoters harboring various CCEs display different circadian phases. The promoters contain one, two, or three tandem copies of D box or Cry1proD element, which were inserted into the P(SV40)-Luc vector to generate an array of reporter constructs.

(B) The 336 bp of *Cry1* intron sequence confers phase delay to D box and Cry1proD element. Reporter constructs were generated similarly as in (A) except that the 336 bp of *Cry1* intron sequence was inserted. The experiment was performed as in Figure 1A (A and B).

(C) The measured phases conferred by the composite promoters are consistent with those predicted by phase vectors. (Left) The phase vectors of oscillations driven by various promoters without the intron sequence (colored arrows) and those driven by the intron sequence (~CT17.5) or P(Cry1) (~CT10) (two black arrows) are plotted with summed phase vectors (center of colored ellipsoidal disks). The ellipsoidal disk represents 95% confidence region. (Right) The summed phase vectors in the left circle are plotted with phase vectors of measured oscillations driven by the composite promoters. (Rightmost) The predicted phases from the simple phase-vector model are plotted against the observed phases. Error bars represent SD (n = 3).

(D) *Cry1* rescue of circadian oscillation in *Cry1*^{-/-}:*Cry2*^{-/-} cells using synthetic composite promoters. The composite promoters presented in (B) were used to drive *Cry1* expression. *Cry1*^{-/-}:*Cry2*^{-/-} cells were cotransfected with a *Cry1* expression construct and a P(*Per2*)-dLuc reporter.

(E) Prolonged phase delay of *Cry1* expression correlates with period length of rescued oscillations. The period lengths of rescued oscillations are plotted against the phase delay of various composite promoters' activity relative to P(Cry1)-*Cry1* intron 336-Luc activity presented in (B). Mean and SD (error bar) of two independent experiments are shown (each experiment contains three samples; n = 3).

Data are representative of two independent experiments (A, B, and D). See also Figure S6, Table S2, and Table S3.

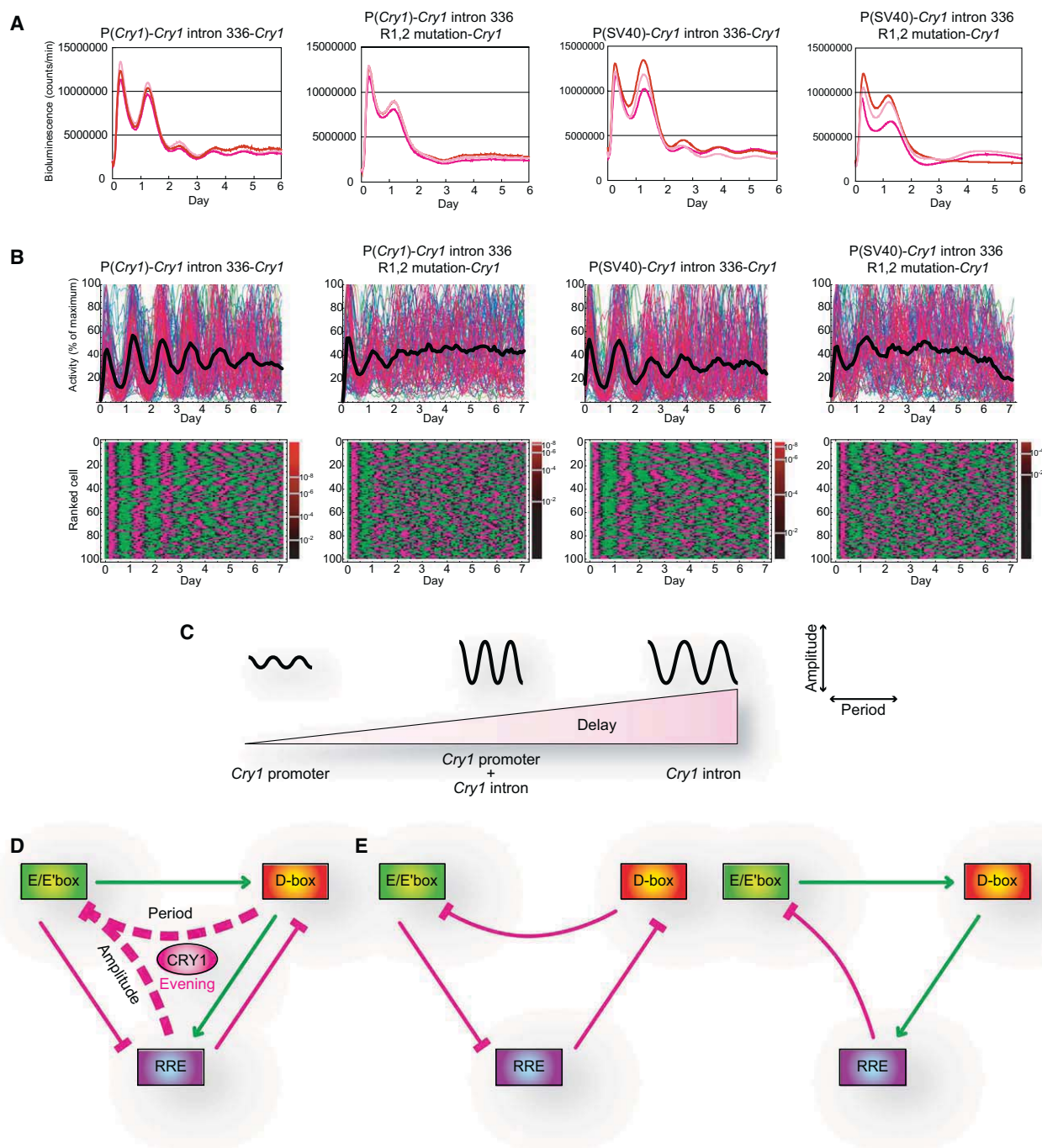


Figure 7. Single-Cell Analysis Confirms the Requirement of *Cry1* Phase Delay

(A) P(*Per2*)-dLuc bioluminescence levels in transfected *Cry1*^{-/-}:*Cry2*^{-/-} cells as recorded. The P(*Per2*)-dLuc reporter and a *Cry1* expression construct as indicated were cotransfected into *Cry1*^{-/-}:*Cry2*^{-/-} cells, and bioluminescence expression was recorded with a PMT. Data from three independent samples are shown.

(B) P(*Per2*)-dLuc bioluminescence levels in transfected individual *Cry1*^{-/-}:*Cry2*^{-/-} cells as recorded by a luminescence microscope (n = 100). Reporter activities from each cell were normalized so that the maximum and minimum bioluminescence values are 100% and 0%, respectively. The mean reporter activity for all of the analyzed single cells at each time point is indicated by a thick black line (top row). Time series of bioluminescence expression shown in the top row were redrawn as heatmaps (bottom row). Each row in the heatmap represents a time series of P(*Per2*)-dLuc reporter activities from a single cell. The corresponding p value of rhythmicity at the period of maximum autocorrelation was evaluated for each time series and is depicted on the right. One-hundred cells were randomly selected and individually analyzed. Data are representative of two independent experiments (A and B).

(C) The roles of phase delay in *Cry1* expression. Through regulation of *Cry1* expression, the promoter and intron primarily affect the amplitude and period of the clock system, respectively.

Cry1-rescued *Cry1*^{-/-}:*Cry2*^{-/-} cells at the cell population level, which lends strong support for our finding that delay of *Cry1* expression is required for circadian clock function.

DISCUSSION

Cry1 Phase Control Mechanism

In this report, we provided experimental data, as well as model predictions, for a “combinatorial regulatory mechanism” to explain the delayed expression of *Cry1*. We newly identified D boxes, which overlap with the E/E' box and confer phase delay over E/E' box activity. In addition, we also demonstrated that the previously identified RREs in the first intron (Ueda et al., 2005) can confer the additional phase delay in *Cry1* expression. Furthermore, we observed that the synthetic pure RREs, in combination with *Cry1* promoter, rescued circadian rhythmicity in *Cry1*^{-/-}:*Cry2*^{-/-} cells with statistical significance ($p < 0.01$; Figure S5D). Together with the observation that the combination of pure D box and *Cry1* intron sequence also rescued rhythms (Figure 6D), we conclude that the RRE and D box elements can recapitulate the basic function of *Cry1* intron and *Cry1* promoter, respectively. It should be noted that the possible contribution from unknown elements in *Cry1* intron 336 sequence could not be completely excluded because the relative amplitude of the pure RRE elements (combined with *Cry1* promoter) is slightly lower than that of the wild-type *Cry1* intron 336 (combined with *Cry1* promoter, Figure S5D).

General Design Principles for New Phases

As revealed in this study, these multiple distinct regulatory sites (i.e., two RREs in first intron and the E/E' box and D boxes in the promoter region) function in a coordinated fashion to generate substantial phase delay, leading to evening-time expression. Interestingly, in an effort to study design principles of the circadian clockwork, we employed a simple phase-vector model in which the new evening-time could be predicted by the combination of two component phase vectors. Although the phase-vector model was not used for phase prediction in our previous study (Ukai-Tadenuma et al., 2008), such a model is also applicable to this previous study when we take into account the time delay associated with transcription/translation of regulator proteins and the *Luciferase* reporter ($r^2 = 0.99$, $p < 0.01$; Figures S7A and S7B and Table S4). These results show that a new phase can be generated through combinatorial synthesis of either two transcriptional regulators or two clock-controlled DNA elements and also can be described, at least to a first-order approximation, by a phase-vector model. Taken together, this combinatorial regulatory mechanism for the generation of new circadian phases of transcription represents a general design principle underpinning the complex system behavior.

Although the phase-vector model predicts the phase of a synthesized oscillation, it is only a first-order approximation.

For example, there are some discrepancies between predicted and measured amplitudes. Also, the wave form generated by the combination of E' box-driven promoter and the *Cry1* intron sequence appeared like a “two-peak” wave form (Figure S4, upper-left), indicative of nonlinear effects or involvement of yet unknown factors. In detailed analysis, we found that the ~11 hr period oscillation was actually a significant component in the two-peak wave form (Figure S4, upper-right; $p < 0.01$). This is the first demonstration of synthesized ultradian rhythms, which may lead to insights into mechanisms of ultradian gene expression with harmonic periods, as recently reported (Hughes et al., 2009).

Delayed *Cry1* Expression Contributes to Clock Robustness

In this study, we focused on the level of transcriptional regulation and demonstrated the importance of delay in feedback repression at the intracellular level. Constitutive expression of *Cry1* abolished circadian rhythmicity in wild-type cells (Ueda et al., 2005) and failed to restore circadian oscillation in arrhythmic *Cry1*^{-/-}:*Cry2*^{-/-} cells (Figure S6D and Figure 7A), suggesting that rhythmic expression of *Cry1* is important for clock function. We revealed that the transcriptional oscillation of *Cry1* with a correct phase with substantial delay was sufficient and required to rescue circadian oscillation in arrhythmic *Cry1*^{-/-}:*Cry2*^{-/-} cells (Figure 5) and that transcriptional oscillation of *Cry1* with a prolonged delay slows circadian oscillation (Figure 6). Importantly, we also confirmed that the amount (baseline) of CRY1 protein was not responsible for the changes in amplitude (Figure S5A) and period (Figure S6E) of rescued oscillations. These results suggest that the phase of *Cry1* expression is responsible for the changes in rescued amplitude and period rather than the amount (baseline) of CRY1 protein. Because we confirmed the significant linear correlation between transcriptional activities and protein levels ($p < 0.01$), when monitored by firefly *Luciferase*, and CRY1 protein amounts, when monitored by fusion *Renilla Luciferase*, irrespective of cell types ($r^2 = 0.93$ in NIH3T3 and $r^2 = 0.90$ in *Cry1*^{-/-}:*Cry2*^{-/-} cells), we speculated that phase of CRY1 protein level would be responsible for the amplitude and period of rescued oscillations.

It should be noted that the CRY1 protein expression levels in our experiments are within a certain range (Figure S5A and Figure S6E), and we do not exclude (and our current results are not in conflict with) the notion that CRY1 protein amounts may affect the parameters of clock function when CRY1 protein levels drastically differ from those in our experimental system, as previously reported (Baggs et al., 2009; Ueda et al., 2005). In addition, we do not exclude the possibility that other regulatory mechanisms such as posttranscriptional modifications (Lee et al., 2001; Liu et al., 2008) play important roles in attaining the robustness of the clock. For example, rhythmic expression of PER2 is recently reported to play a prominent role in CRY1 function (Chen et al.,

(D) A schematic diagram of a minimal circuit for the mammalian circadian transcriptional network. The network can be represented by a simple circuit, consisting of two transcriptional activations (green arrows) and four transcriptional repressions (red arrows) on three regulatory elements (three rectangles).

(E) The minimal circuit envisaged as a composite of two distinct oscillatory network motifs: (1) A repressilator that is composed of three repressions (left) and (2) a delayed negative feedback loop, which is composed of two activations and one repression (right). See also Figure S7, Movie S1, and Table S3.

2009). And PER2 is phosphorylated by CKI δ/ϵ , which is also an essential mammalian clock component (Lee et al., 2009) and has been recently implicated in temperature compensation (Isojima et al., 2009). In addition, it has been reported that constant supply of membrane-permeable CRY1 and CRY2 proteins rescued circadian oscillation in *Cry1*^{-/-}:*Cry2*^{-/-} cells (Fan et al., 2007). In line with this observation, our single-cell analysis indicated that a fraction of individual cells transfected with *Cry1* driven by a constitutive promoter exhibited weak circadian oscillation even though the rhythms are rather transient (Figure 7B), implying that constant *Cry1* expression might partially rescue circadian clock function. This qualitatively less-robust clock function is probably attributable to posttranscriptional and post-translational mechanisms (Lee et al., 2001; Liu et al., 2008).

Even in this context, it appears that phase delay in rhythmic *Cry1* expression may contribute to the robustness of clock function by ensuring properly timed nuclear translocation of CRY proteins. This idea is strongly supported by our results presented in this study: delayed *Cry1* expression via D box-mediated transcription (i.e., from *Cry1* promoter) allowed partial rescue, and further delay via the RREs from the *Cry1* intron restored circadian rhythmicity with amplitude and persistence comparable to wild-type cells.

Design Principle for a Circadian Transcriptional Network

Delayed feedback repression is one of the most prevailing but as yet unverified design principles for a circadian transcriptional network. This design principle predicts that decreased delay dampens circadian oscillations and that prolonged delay slows down circadian oscillations (Figure 7C, Figures S7C–S7F, and Extended Experimental Procedures) (Bernard et al., 2006; Lewis, 2003; Novak and Tyson, 2008). The results presented in this study are consistent with the two predictions from the delayed feedback repression, suggesting that it is an applicable design principle in the mammalian circadian transcriptional network.

A Minimal Circuit for a Circadian Transcriptional Network

In a previous effort to identify a minimal circuit of the complex autoregulatory transcriptional networks in the mammalian circadian clock, we showed that day-time promoter activity can be reconstructed by combining a morning-time activator and a night-time repressor and night-time promoter activity by combining a day-time activator and a morning-time repressor (Ukai-Tadenuma et al., 2008). In this study, we succeeded in synthesizing the evening-time phase control of transcription. Our previous and current results suggest that the complex mammalian transcription network can be reduced to a relatively simple diagram (Figure 7D) that would consist of three regulatory elements and six transcriptional regulations (two activations and four repressions). It is noteworthy that this diagram can be envisaged as a composite of two distinct oscillatory network motifs (Figure 7E). The first oscillatory network motif is composed of three repressions (i.e., E/E' box to RRE, RRE to D box, and D box to E/E' box), comprising a cyclic negative feedback loop—a repressilator (Elowitz and Leibler, 2000). The second oscillatory network motif is composed of two activations (i.e.,

E/E' box to D box and D box to RRE) and one repression (i.e., RRE to E/E' box), comprising a delayed negative feedback loop. It is interesting to note that oscillatory properties of both network motifs were experimentally suggested by synthetic approaches (Elowitz and Leibler, 2000; Stricker et al., 2008). Therefore, further experimental and theoretical analyses of the composite of these oscillatory network motifs lie ahead.

EXPERIMENTAL PROCEDURES

Preparation of Embryonic Fibroblasts from *Cry1*^{-/-}:*Cry2*^{-/-} Double-Knockout Mice

Cry1^{-/-}:*Cry2*^{-/-} double-knockout mice (van der Horst et al., 1999) were carefully kept and handled according to the RIKEN Regulations for Animal Experiments. The dissociated cells (mouse embryonic fibroblasts [MEF] from *Cry1*^{-/-}:*Cry2*^{-/-} double-knockout mice; *Cry1*^{-/-}:*Cry2*^{-/-} cells) were suspended and cultured in DMEM (Invitrogen) supplemented with 10% FBS (JRH Biosciences) and antibiotics (see Extended Experimental Procedures for details).

Real-Time Circadian Reporter Assay Using NIH 3T3 Cells and *Cry1*^{-/-}:*Cry2*^{-/-} Cells

Real-time circadian assays were performed as previously described (Sato et al., 2006; Ueda et al., 2005) with the following modifications. NIH 3T3 cells were transfected with the *Luciferase* reporter plasmids. *Cry1*^{-/-}:*Cry2*^{-/-} cells were transfected with pGL3-P(*Per2*)-dLuc reporter plasmid (Sato et al., 2006) and each *Cry1* gene expression vector. The cells were stimulated by 10 μ M (NIH 3T3) or 30 μ M (*Cry1*^{-/-}:*Cry2*^{-/-} cells) forskolin (Fermentek), and the bioluminescence was measured at 30°C (see Extended Experimental Procedures for details).

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H.R.U. and A.C.L. designed the research scheme. M.U.-T. constructed most of materials and performed real-time luminescence assays. R.G.Y. conducted bioinformatic, statistic, and theoretical analyses. H.X. constructed the virus vector and performed the corresponding real-time luminescence assays. J.A.R. performed ChIP analysis and mRNA accumulation analysis. All authors discussed the results and commented on the manuscript text.

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